ENDOPHYTIC BACTERIA AND FUNGI FROM EDIBLE BAMBOO SHOOTS OF KARNATAKA

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GENERAL INTRODUCTION

In nature, microbes are widely distributed. De Bary used the term "endophyte" in 1866 to distinguish organisms that reside inside host plants Endophytes such as bacteria, fungus, and actinomycetes may live fully or partial life time in a host without producing obvious damage or infectious diseases, these endophytes can colonise healthy leaves, petioles, stems, twigs, bark, roots, fruit, flowers or seeds inside the plant tissues. They can also form mutual relationships with the host plants (Petrini, 1991)

Bamboos are large perennial grass distributed widely from tropical and subtropical zones, belonging to the family Poaceae. Bamboo shoot is used as a traditional delicacy in many countries including India as they are low in fat, high in potassium, carbohydrate, dietary fibres, vitamins and active materials. Bamboo shoots are consumed in raw, canned, boiled, marinated, fermented, frozen, liquid and medicinal forms. According to the consumption pattern of bamboo shoots in most of the countries is traditional, non-standardized, seasonal and region-specific (Choudhury *et al*., 2012). Three types have been identified: Arundinarieae, which are temperate woody bamboos; Bambuseae, which are tropical woody bamboos; and Olyreae, which are herbaceous bamboos (Ahmad *et al*., 2021).

In India, 18 native bamboo species have been identified as economically important of which around 10 species are edible. In China, shoots have been exploited as a traditional forest vegetable for more than 2,500 years and has been explicitly used in the traditional South Asian cuisine for many centuries. *Phyllostachys pubescens* (Moso) has been largely cultivated and utilized for shoots in China. New culms or juvenile shoots of bamboos usually develop with the beginning of the rainy season in June or July (Chandramouli *et al.*, 2014).

In Asian countries, different species and different parts of bamboos have been used for building material, handicraft article, food material and traditional medicine. An extract of bamboo, is used as an active element to inhibit inflammation in the throat. Some biologically active components in bamboo and their potential health benefits have been widely studied (Tripathi and Anjum, 2015).

In the last years, few studies have been conducted on the antipathogenic activity of the endophytic community of this plant. The aim of the present study is to explore the diversity of culturable endophytes from Bamboo shoots and to assess a promising source of potential bioactive compounds which have antimicrobial property.

Bamboo is a vital plant, and its mingling with the indigenous population suggests that new endophytic fungal strains may be discovered, which could lead to the discovery of novel natural products (Giba *et al*., 2020). A wide range of bioactive secondary metabolites with distinct structures, such as alkaloids,

xanthones, benzopyranones, tetralones, flavonoids, terpenoids, phenolic acids and quinones, have been extracted from endophytes (Tan and Zou, 2001).

REVIEW OF LITERATURE

Most of the microorganisms that inhabit plants play a major role in the plant's health and development, although, sometimes they are neutral It is considered that a single plant species could possess thousands of microbes, categorized as epiphytes (microbial inhabitants of the rhizosphere and phyllosphere; those near or on plant tissue) or endophytes (microbes residing within plant tissues in leaves, roots or stems), depending on their area of colonization in the plant species. Endophytic fungi are common and diverse, and living asymptomatically within plant tissues or organs. The relationship between the endophytic fungi and their host plant is complicated (Saikkonen *et al.,* 1998; Azevedo *et al.,* 2000).

Nunes states that Endophytic bacteria have been found in virtually every plant studied. Diverse array of endophytic bacteria which forms a non-pathogenic relationship with their hosts also confer benefit to plants (Nunes and Melo, 2006).

Endophytes are reported to produce a number of bioactive metabolites in a single plant or microbe which served as an excellent source of drugs for treatment against various diseases and with potential applications in agriculture, medicine, food and cosmetics industries These secondary metabolites were categorized in to various functional groups, alkaloids, benzo pyranones, chinones, flavonoids, phenolic acids, quinones, steroids, saponins, tannins, terpenoids, tetralones, xanthones, and many.

(Bahig El-Deeb, Khalaf Fayez *et al*., 2012) have made a study on the diversity and beneficial characteristics of endophytic microorganisms from *Plectranthus tenuiflorus.* Endophytic bacteria were isolated from root, stem, and leaves of *P. tenuiflorus* plant. The isolates were identified by partial sequencing of their 16S rRNA gene. The isolated endophytic bacteria were *Bacillus sp., Bacillus megaterium, Bacillus pumilus, Bacillus licheniformis, Micrococcus luteus, Paenibacillus sp., Pseudomonas sp.,* and *Acinetobacter calcoaceticus.* Considerable antimicrobial activities against a panel of human pathogenic microorganisms (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus agalactiae, Proteus mirabilis, and Candida albicans*) were recorded using crude extracts of the collected endophytic strains. The exploited bacteria isolated from *P. tenuiflorus* showed the antimicrobial and enzymatic activities.

Mohammed and Bahgat they worked on isolation and visualizing of endophytic bacteria in its natural niche, inside plant tissue, and identification of cultivable endophytic bacteria that colonize aerial parts of *Capparissinaica,* as an indigenous plant to Sinai-Egypt. The morphological, biochemical and 16Sr

RNA gene sequence analysis revealed that Bacillus species were the most dominant endophytic bacteria. Mixed microbial culture system of isolated endophytic bacterial species was used for production of secondary metabolites paper Chromatography and HPLC analysis showed that bacterial extract produced quercetin and quercetin 3-O-glucopyranoside which were detected in plant extract. Hence, it was concluded that endophytic bacteria produced flavonoid compounds. (Magdymohsen Mohammed Bahgat, Mona Mohamoud *et al*., 2014)

A study was conducted on walnut tissue using tissue isolation technics, 64 strains of endophytic fungi were isolated from walnut tissues (Wang *et al*., 2020).

Anil had evaluated Preliminary phytochemical screening of solvent extracts viz. methanol and ethyl acetate of fermented *Bambusa balcooa* shoots were carried out. Study reveals the presence of tannins, steroids, phenols, glycosides, flavanoids, carbohydrates and proteins. Further, the therapeutic potential was confirmed by the antimicrobial property of the methanolic extracts against 4 bacterial strains viz., *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli* and 3 fungal strains viz*., Aspergillus niger, Candida albicans* and *Fusarium oxysporum*. Maximum activity (Anil Singh *et al*., 2012)

Study was carried out by Joseph to characterize the bioactive constituents present in the leaf extracts of certain bamboo species using Fourier transform infrared spectroscopic analysis (FT-IR). The various solvent extracts of various bamboo leaves subjected to qualitative analysis showed the availability of phytoconstituents including alkaloids, phenolic compounds, flavonoids, saponins, glycosides, terpenoids, steroids, coumarins and phytosterols. Quinones were completely absent in all the five bamboo species.. The present investigation has also opened avenues for further research into the development of potent phytomedicines from different bamboo species. (Joseph *et al.,* 2014).

Chandrawali evaluated Bamboo shoots of four different species for their antibacterial activity, total phenol content, flavonoid content, antioxidant activity and total phytochemical analysis. The fresh shoots were extracted successively by using various solvents such as petroleum ether, ethyl acetate, methanol and water. The antioxidant and antimicrobial properties were investigated with the extracts. The antioxidant activity of the extracts was evaluated by DPPH assay, FRAP assay, Linoleic acid autooxidation and Hydrogen peroxide radical scavenging activity. The volatile components present in the extracts were analyzed by GCMS-MS technique. A number of compounds with important medicinal properties were detected in the extracts. The antimicrobial activity against gram positive and gram negative bacteria was evaluated by agar well method. All the extracts possessed antimicrobial activity against both *S.aureus , K. pneumonia* bacteria. The antioxidant activity was found to be highest in methanol extracts of all the four bamboo species. The results indicate that these

bamboo shoots are rich sources of polyphenols, flavonoids and terpenes with potential antioxidant and antimicrobial activity.(Chandrawali *et al.,* 2016).

Few report States that Pathogen growth inhibition by endophytic fungi. In a study seven endophytic species with more vigorous growth on the media were selected for dual cultures with *Monilia laxa* and *Agrobacterium tumefaciens*. As it was demonstrated by results, all species showed a range of inhibition and growth factors of pathogens in all plates were collectively impaired in compare with controls. In all plates, endophytic fungi had higher radial growth rate and their confrontation with pathogens had not only no influence on colony expansion and development but also in the case of some species induced improved growth rate to the endophyte.

With 23 genera and 125 species of bamboo, both native and foreign, India is the world's second-largest producer of bamboo (Verma and Bahadur, 1980). According to Hore (1998), the North Eastern region of India alone is home to a significant diversity of bamboo, with 19 genera and 78 species. Bhuyan *et al*. (2007) states that Arunachal Pradesh has the highest concentration of bamboo resources in the nation, with 57 species. This part of India is a symbol of the continued connection to traditional uses and local knowledge on the management and use of bamboo species.(Giba *et al*., 2020)

RESEARCH OBJECTIVES

1. **To isolate, identify and characterize the Endophytic Bacteria and Fungi from edible Bamboo shoots.**
2. **Antimicrobial activity of Endophytic Bacteria and Fungi against human pathogens.**
3. **Molecular identification of selected Endophytic Bacteria and Fungi from edible Bamboo shoots.**
4. **Antioxidant activity and Phytochemical analysis of Endophytic Bacteria and Fungi extracts.**
5. **To characterize the Bioactive compounds of Endophytic Bacteria and Fungi of edible Bamboo Shoots.**

CHAPTER I

Isolation, Identification and Characterization of Endophytic Bacteria and Fungi from Edible Bamboo Shoots.

1.1 INTRODUCTION

Entophytic bacteria colonize enormous number of plants in the healthy tissue which are not causing injury to the host and symbiosis associate with the plant. The word "endophytes" was first used by scientist De Bary in 1866 to describe microorganisms found in plant tissues (Stone et al., 2000). Bacteria colonize in all part of plants leaf ,stem, roots, fruits (Haruna *et al*., 2018).

The young, edible bamboo plants that have just sprung from the earth during the rainy season are known as bamboo shoots (Karthik and Jayashankar, 2023). The freshly harvested shoot is cream yellow in colour, has a strong smell and tastes sweet. Bamboo shoots are a delicacy, a nutritional dish of many countries However, all species of bamboo shoots available worldwide are not edible. Bamboo shoots have excellent anti-microbial qualities(Park and Jhon, 2010).

Use of antagonistic endophyte as Biocontrol compounds are a viable option for managing some plant diseases with no negative environmental impact (De Silva *et al*., 2019). The bacteria, fungus, and actinomycetes that make up the plant microbiome include bacterial endophytes, which can invade living plant tissues or intracellular cells without harming the host plant (Taulé *et al*., 2021). Endophytic bacteria have many benefits, increasing the production of mineral absorption, increasing plant growth by producing phytohormones, for survival in extreme climate condition, nitrogen fixation and increasing plant defence to bacterial and fungal diseases (Triwidodo and Listihani, 2021)

Bamboo shoots that have been fermented contain a wealth of microorganisms, many of which are probiotics. They provide a variety of health advantages when taken, including antioxidant and anti-cancer properties, blood pressure reduction, heart disease prevention, and weight loss, to mention a few. In addition to these, they can be used in several industries, including the food and pharmaceutical industries field (Behera and Balaji, 2021).

1.2 MATERIALS AND METHOD

1.2.1 Collection of Plant sample:

Bamboo shoots were collected randomly from different region of Karnataka. The healthy Bamboo shoots were selected to avoid interfering by plant pathogens. Collected Bamboo shoots are brought to the laboratory within 24 h in a cleaned sterile polythene bag.

1.2.2 Isolation and identification of endophytic bacteria from edible bamboo shoots.

Sample processing for Isolation of endophytic bacteria:

A. *Pre-treatment of plant shoots*

Bamboo shoots samples were collected and the shoots were cut into about 1cm long segments/pieces and then washed in running tap water for 10 min to remove the soil particles, microbes and adhered debris, then again washed in tap water and finally washed with distilled water.

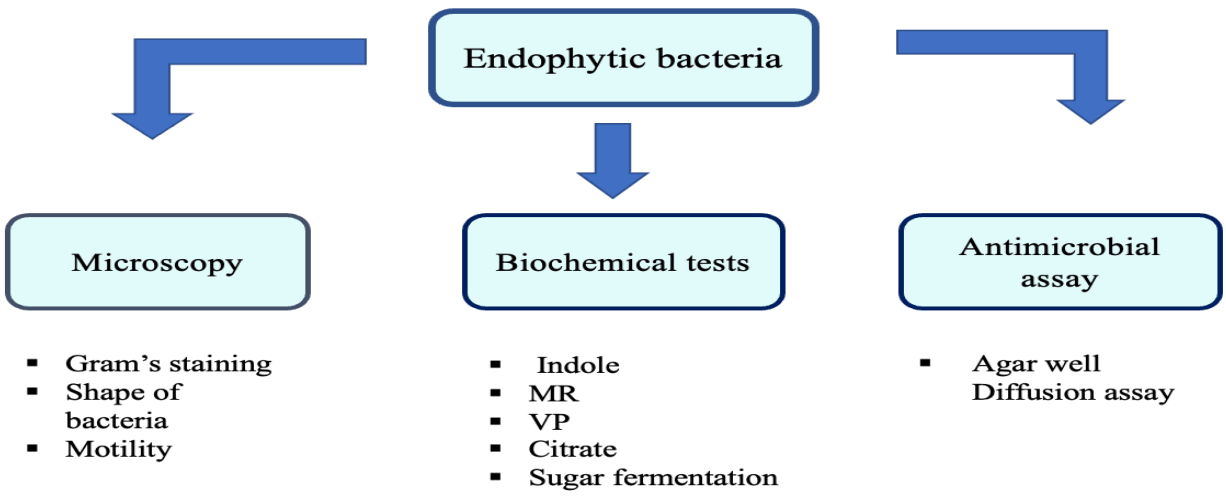
B. *Surface Sterilization*

Surface sterilization was done by using the protocol described by (Petrini, 1992; Werner *et al*., 1997) with few modifications. Plant samples were immersed 2 times in 70% ethanol for three min and immersed twice in 2% aqueous solution of Sodium hypochlorite for 5 min and again immersed for 1 min in 70% ethanol. Finally, rinse the plant samples 6-8 times with sterile water for 5 min and wash 2 times in sterile distilled water for 5 min to remove the surface sterilization agents with further drying in sterilized paper in laminar air flow hood.

C. *Sterility Check*

To confirm that the plant surface were effectively decontaminated sterile distilled water that was used in the final rinse of surface sterilization procedure were plated on the Nutrient agar medium (Mclnroy and Kloepper 1994) and incubated at 37ºC for 24h. Bacterial growth was observed after 24 h and also checked out for possible growth (Hallman *et al*., 1999)

Workflow for bacterial identification



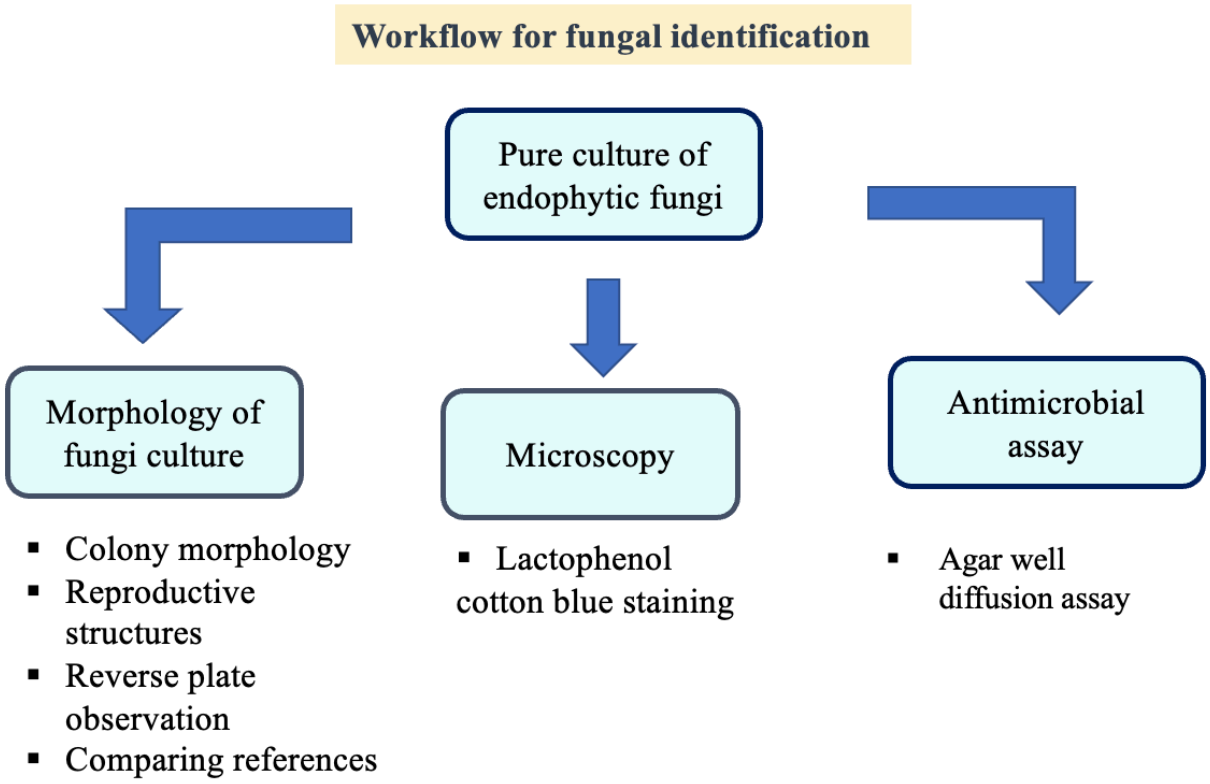
Nutrient agar media preparation for isolation of endophytic bacteria

Peptone -5.0gm
Beef extract- 3.0 gm
Agar – 15.0 gm
Distilled Water- 1000 ml
Anti-fungal agent - Fluconazole (100µg/mL) to suppress Fungal growth

1.2.3 Isolation and identification of endophytic fungi from edible bamboo shoots.

Isolation of Endophytic Fungi from bamboo shoot.

The collected Bamboo shoot sample are washed under running tap water to remove dirt on the surface, then cut into small size segment and sequential rinsing with 70% ethanol for 30 seconds, and 0.5% sodium hypochlorite for 1 minute followed by sterile distilled water for 2-3 times and dried using sterile Whatman no. 1 filter paper The dried shoot segments were placed on Petri dish containing Sabouraud Dextrose Agar (SDA) media and allowed to grow in room temperature. Plates were observed periodically and when hyphae appeared out from plant segments they were sub cultured and brought to pure culture in PDA slants and stored at 4ºC. All isolated endophytic fungi are maintained in the refrigerator. (Okuda *et al*.,2005 ; Petrini,1991; Basha *et al*.,2012)



Sabouraud Dextrose Agar media preparation for isolation of endophytic fungi

Peptone – 10 gm
Dextrose- 40 gm
Agar- 20gm
Distilled Water- 1000 ml
Antibiotic - Streptomycin (100µg/mL) to suppress bacterial growth

1.3 RESULT

A total of 137 endophytic bacterial isolates were isolated from 8 different Bamboo shoots collected from 10 districts of Karnataka.

There are total 77 bacilli and 60 cocci total 97 gram positive and 40 gram negative

71 endophytic fungi cultures were isolated from the 8 species of Bamboo shoots collected from 10 districts of Karnataka.

A total of 71 isolates were subjected to fungal staining by using cotton blue. Microscopic observations such as *Aspergillus species, Fusarium species, Alternaria species, Penicillium species, Trichoderma species* were mainly reported.

1.4 DISCUSSION

Biotic and abiotic factors cause an enormous amount of yield and economical loss. However, endophytes can play a significant role in enhancing the tolerance of plants. Endophytes systematically colonize different parts of the host, but plants use a variety of defence mechanisms towards microbial infection. However, they have to survive the oxidative environments (Mengistu, 2020) Microorganism identification. Microorganisms such as bacteria, fungus, grow in distinct environmental conditions and their composition is changed by impacts of numerous environmental elements. Variations in the physiology of microorganisms lead to their growth with distinct morphological characteristics under varying environmental conditions. Traditional methods and tools are the gold standard for characterising and identifying microorganisms. (Missoum, 2020) Isolation of endophytes are made effective with modification of sterilization protocol (Schulz *et al.*, 2006)

CHAPTER II

Antibacterial and Antifungal activity of Endophytic Bacteria and Fungi against Human Pathogens

2.1 INTRODUCTION

Endophytes are microorganisms that are present in surviving tissue of plants establishing mutual relationship. These endophytes defend their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Carroll and Carroll, 1978; Strobel, 2002). Endophytes are rich sources of bioactive metabolites, which have important in medicine, agriculture and food industries (Tan and Zou, 2001). These metabolites, when isolated and characterized, it can be used in industries, agriculture and in medicines (Kumar *et al,* 2014). The relation between fungal endophytes and host plant is enormous and different, which is range from symbiotic relationship to antagonistic relationship (opportunistic pathogenic) (Schulz and Boyle, 2008). They develop the resistance of host plants to adverse conditions by discharging bioactive metabolite. These metabolites belong to the category of secondary metabolites of plants which include alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones and xanthones (Tan and Zou, 2001). The innovation of novel anti-microbial, anti-cancerous, anti-oxidant, insecticidal and immune modulatory metabolites from endophytes is a significant alternative to overcome the increasing levels of drug resistance by human pathogens (Song, 2008; Demain and Sanchez, 2009). In association to the plants, endophytic fungi can be cultured promptly and sufficient biomass can be accumulated by large scale fermentation. Production of bioactive compounds can be increased by genetic engineering of endophytic fungi in order to meet demands while keeping biodiversity and sustainable ecosystem.

An antimicrobial compound is an agent that kills Microorganisms or stops their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotics are used against bacteria, and antifungals are used against fungi. Antimicrobial use has been common practice for at least 2000 years. Ancient Egyptians and Greeks used specific plant extracts to treat infection. The literature review confirms that endophytes can produce antimicrobial compounds

For these above reasons, effort was made to test the endophytes obtained from bamboo shoots for the production of any antimicrobial compounds against 10 bacterial pathogens and two fungal pathogens.

2.2 MATERIALS AND METHOD

Test culture and growth condition for endophytic bacteria

The given bacterial cultures were grown fresh in Luria Bertani (LB) media at 37 ^{0}C for 24 hrs. The culture was centrifuged at 10,000 rpm for 10 mins to collect the cell free supernatant (CFS) and filter sterilized which was then used for antibacterial assay.

PART A – Preparation of Cell-free Supernatant for antimicrobial assay

Test Culture and Growth Condition of Endophytic Bacteria:

The test Endophytic bacterial cultures were grown fresh in Nutrient broth (NB) at 37°C for 24 h. later centrifuged for 10 min at 10,000 rpm. Cell-free supernatant (CFS) were collected and filter sterilized which was then used for antimicrobial assay.

Test culture Fermentation and mass production of Endophytic fungi:

For the production of secondary metabolite, endophytic fungal cultures were grown in Sabouraud Dextrose Broth (SDB) by placing agar blocks of actively growing pure culture (8mm diameter discs) in 250 ml Erlenmeyer flask containing 100 ml of SDB and incubated at 28±1ºC for 14 days. The culture was centrifuged at 10,000 rpm for 10 min to collect the cell-free supernatant (CFS) and was filter sterilized which was then used for antimicrobial assay.2.5% DMSO is taken as negative control.

NOTE- while culturing no antibiotics and antifungal agents are not added.

PART B- Preparation of bacteria and fungi pathogens culture

Pathogenic bacterial cultures and growth condition:

The pathogenic cultures listed below were cultivated for 24 hours at 37°C with continual shaking at (150 rpm) in Brain Heart Infusion (BHI) medium.

Pathogenic fungal cultures and growth condition:

The pathogen culture *Aspergillus niger* and the yeast *Candida albicans* were grown in SDB at 35 °C for 48 hrs.

Antibacterial activity by agar well diffusion method:

- BHI agar plates were prepared by inoculating freshly grown pathogenic culture.
- Well of 6 mm in diameter was punched plate by using sterile cork borer
- Then 70µl of given CFS of test culture was added in each well Later, plates are incubated at 37°C for 24 to 48 hour.
- Antibiotic streptomycin was added as positive control and sterile nutrient broth as negative control.
- After incubation zone of inhibition was measured in mm and recorded the results are expressed as mean ± standard error of mean of three replicate. Mean differences in inhibition zones were evaluated with ANOVA.

Antifungal activity by agar well diffusion method:

- SDA agar plates were prepared by inoculating freshly grown fungl pathogenic culture.
- Well of 6 mm in diameter was punched plate by using sterile cork borer
- Then 70µl of given CFS of test culture was added in each well Later, plates are incubated at 35°C for 24 to 48 hour.
- Antifungal fluconazole was added as positive control and sterile SDB media as negative control.
- After incubation, the zone of inhibition was measured in mm and recorded the results are expressed as mean ± standard error of mean of three replicate. Mean differences in inhibition zones were evaluated with ANOVA.

2.3 RESULTS

Total 137 bacteria isolates and 71 fungi culture crude sample was subjected to antimicrobial assay against clinical pathogens 10 bacteria and 2 fungi pathogens

The clinical pathogens were collected from Medical college KIMC Madikeri. *Escherichia coli, Shigella sonnei, Salmonella typhi, Actinobacter baumannii, Proteus vulgaris, Enteracoccus faecalis, Methicillin resistant Staphylococcus aureus, Methicillin susceptible Staphylococcus aureus* and *Klepsiella pneumonia.* Culture B3 showed high antibacterial activity Against *Escherichia coli, Proteus vulgaris* and *Klebsiella pneumoniae.* pathogen

Culture B3 showed moderate antifungal activity Against *Candida albicans. Aspergillus niger* pathogen. B45 shows high activity against *Methicillin resistant Staphylococcus aureus*. B3, B45, B65, B68 and B115 Endophytic bacteria CFS shows a remarkable activity which shows they have some antibacterial compound in it and B3, B45 and B68 shows moderate antifungal activity. F2, F14, F33, F45 and F62 Endophytic fungi CFS shows a remarkable activity which shows they have some antibacterial compound in it and F14 and F33 shows moderate antifungal activity. The above-mentioned strains of fungi and bacteria were further carried to next sequence of analysis

2.4 DISCUSSION

In this current chapter we had tested the isolated endophytic fungi and bacteria cell free supernatant of antimicrobial property by agar well diffusion method which was a preliminary step for the selection of capable isolate for further study. Antimicrobial assay is focused to find the high impact strains which have the properties to supress the growth of the clinical pathogens. A total of five endophytic bacteria and five endophytic fungi were selected by this method and further analysis was done in detail.

CHAPTER III

Molecular identification of selected Endophytic Bacteria and Fungi from edible Bamboo shoots.

3.1 INTRODUCTION

The techniques for the identification and characterization methods have evolved from classical morphological screening to molecular as well as phylogenetic characterization with gene sequencing analysis. This sequencing analysis targets linear 16S rRNA gene sequences by polymerase chain reaction (PCR) for amplification, DNA-DNA hybridization (DDH) and whole genome shotgun sequencing methods.

The 16S rDNA gene, a barcoding gene used to identify bacteria, was used for identification. In the (NCBI) National Centre for Biotechnology Information site, retrieved sequences are compared with existing sequence using the Basic local alignment search tool (BLAST) programme. The technique of ITS sequencing is used to identify isolated fungus. Bio Edit software is then used to modify and examine the obtained sequences. The obtained ITS region is compared to closely comparable species found in the NCBI database.

3.2 MATERIALS AND METHOD

Molecular identification of bacteria using 16S rRNA Gene Sequence Analysis method:

Pure bacterial culture is grown in LB broth for 24 hrs. Cultures are centrifuged at 15000 g for 1 min and bacterial pellet is collected DNA is isolated by CTAB method. The DNA thus obtained has to be quantified.

Preparation of PCR reaction mixture:

Each PCR reaction for testing the amplification efficiency and development of multiplex PCR assays for DNA barcode primers contained 1µl DNA template (25 ng), 2µl 10X reaction buffer, 0.5µl of MgCl2 (50pM), 1µl of dNTPs mix (10mM), 1µl forward primer (10pM), 1µl reverse primer (10pM), 0.5µl Taq polymerase (5 U/pi) and the final volume 25µl is adjusted with molecular grade water. Marker gene for 16S ribosomal DNA was used.

The PCR used universal primers to amplify approximately 1500 bp. The electrophoresis of the PCR data was followed by purification.

Molecular identification of fungi using ITS Region amplification method:

Nuclear ribosomal internal transcribed spacer (ITS) region are amplified by ITS -1 and ITS - 4 Primer for fungal identification. ITS gene (600bp) amplified from fungus, compared with

100bp ladder, Gel purification protocol, Sanger Sequencing PCR, Post Sequencing and PCR Purification

Data analysis:

The acquired sequences are then altered and analysed using bio edit software. Sequences obtained are compared with existed sequence by using Basic local alignment search tool (BLAST) program from National Centre for Biotechnology Information site (NCBI). Then the results are analysed by using MEGHA 11 software to determine the level of kinship. Phylogenetic trees are created using maximum likelihood and character-based parameter models.

3.3 RESULT

Total 137 Bacterial isolates obtained five prominent species (B3, B45, B115, B65 and B68) reviled significant zone of inhibition against *Escherichia coli, Proteus mirabilis, Klebsiella pneumonia and Shigella sonnei* . These isolates are molecularly identified using 16S rRNA sequencing. phylogenetic tree is constructed results showed that the bacteria is derived from Bacillus species and Gamma proteobacteria. Identified as, *Bacillus licheniformis* (ON254199), *Bacillus velezensis* (ON259692) , *Bacillus subtilis* (ON340723), *Cronobacter sakazakii* (ON479321) and *Pseudomonas otitidis* (0N479241).

Total 71Fungi cultures optained five prominent species (F2, F33, F14, F45 and F62) reviled significant zone of inhibition against *Escherichia coli, Proteus mirabilis. Klebsiella pneumonia* and *Shigella sonnei* . These isolates are molecularly identified using ITS region sequencing. phylogenetic tree is constructed results showed that the culture F2 *Fusarium solani ,* F33 *Aspergillus niger,* F14 *Rigidoporus vinctus,* F45 *Cerrena unicolor* and F62 *Rigidoporus hypobrunneus.*

3.4 DISCUSSION

In the present chapter ten potential isolates which showed good antibacterial activity out of which five endophytic bacteria and five endophytic fungi was taken for further molecular characterization. The endophytic bacteria isolates were identified based on molecular sequencing by 16S rRNA sequences using the BLAST program. The endophytic fungi cultures were identified based on molecular sequencing by ITS region sequences using the BLAST program, Phylogenetic tree is constructed by comparing with strains from GenBank with highest similarities. Phylogenetic tree is constructed using Kimura 2 model with bootstrap method. The neighbour-joining tree and sub tree were generated using MEGA 11 software.

CHAPTER IV

Antioxidant activity and Phytochemical analysis of Endophytic Bacteria and Fungi extracts.

4.1 INTRODUCTION

The emergence of antibiotic-resistant micro-organisms calls for inventive research and development strategies. Inhibition of these pathogenic micro-organisms may be a promising therapeutic approach. The screening of antimicrobial compounds from endophytes is a promising way to meet the increasing threat of drug-resistant strains of human and plant pathogens. The Environment has delivered living organisms with abundant antioxidant molecules. An antioxidant is a stable molecule which provides an electron to a charging free radical and terminates the chain reaction before vital molecules are spoiled. Free radical scavenging property of an antioxidants delays or prevents cellular damage (Halliwell, 1995). Antioxidants may defend the body against Reactive Oxygen Species (ROS) poisonousness either by avoiding the formation of ROS by carrying disturbance in ROS attack, by exchanging them to less reactive molecules or by scavenging the reactive metabolites (Hegde and Joshi, 2009). Synthetic antioxidant like TBHQ (Tertiary- Butylhydroquinone), EDTA (Ethylenediaminetetraacetic acid), α-tocopherol, BHT (Butylated hydroxyanisole) are known to enrich oxidative damages but they have been regulated due to their carcinogenic and dangerous effect on the lungs and liver (Gokhan Zengin *et al*, 2011).

Therefore, the researchers have concentrated on normal antioxidant particularly plant phytochemicals and the microorganisms produce them. Latest exploration reports have shown endophytic bacteria and fungi derived secondary metabolites or bioactive compounds have a great antioxidant activity against foreign elements (Kaul *et al,* 2013).

In the current study, Methanol, Ethyl acetate and Chloroform extracts of all selected endophytic bacteria and fungi were studied for antioxidant potential by using three different assays. All extracts of different endophytic bacteria and fungi exhibited significant antioxidant activity up to a modifiable extent. In this objective, the different solvent extracts of both endophytic bacterial and fungal isolates were evaluated for antioxidant activity by DPPH, Hydrogen Peroxidase and FRAP assay.

4.2 MATERIALS AND METHOD

Cultivation and isolation of extracellular secondary metabolites from selected endophytic Bacteria and fungal cultures

Endophytic Bacteria	Endophytic Fungi
Selected endophytic Bacteria pure cultures are mass cultured for production of secondary metabolites	Selected endophytic fungal pure cultures are subjected for submerged fermentation
Each selected bacteria isolate was freshly sub-cultured and then transferred into 1000 ml Erlenmeyer flasks containing 500 ml of autoclaved Nutrient broth (NB).	Each fungal culture was freshly sub-cultured and then transferred into 1000 ml Erlenmeyer flasks containing 500 ml of autoclaved Sabouraud Dextrose Broth (SDB).
The flasks were then incubated at 37 ^{0}C under continuous shaking conditions for 24-48 hrs in a shaking incubator.	The flasks were then incubated at room temperature under continuous shaking conditions for 21 days in a dark chamber.

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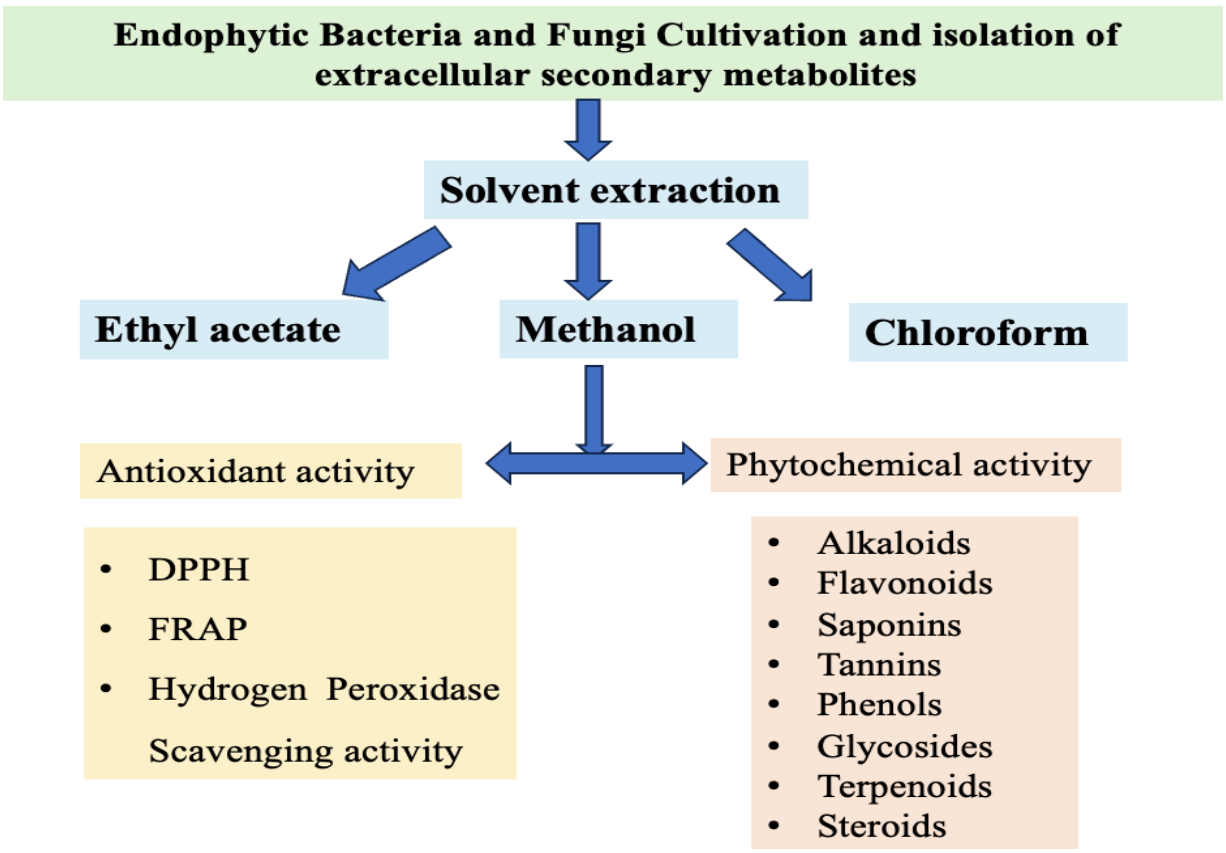


Phytochemical Qualitative assay

1. Determination of Alkaloids

3 ml each extract was stirred with 3 ml of 1% HCl on steam bath. Dragondroff's reagent was then added to mixture. Turbidity of the resulting precipitate was taken as an evidence for the presence of alkaloid.

2. Determination of Flavonoid content

Lead acetate test

1ml of the extract was mixed with 1ml of 10% lead acetate solution and observed for a yellow-colored precipitate in the determination of flavonoid in both endophytic bacteria and fungi samples (Kim *et al*, 2003).

3. Determination of Saponins

Foam test

The extract of 2 ml mixed with 2 ml of distilled water was shaken vigorously and persistent foam was observed and no foam was obtained hence, it indicates the absence of Saponins.

4. Determination of Tannins

Tests for Tannins

Ferric chloride test- In a test tube 2 mL of aqueous extract and 1-2 drops of 5% aqueous ferric chloride solution was added. A bluish black colour which disappears on addition of a few mL of sulphuric acid indicated the presence of tannins.

5. Determination of Phenols

Gelatine test- Gelatine solution (1%) containing 10% sodium chloride was added to 1ml of extract and observed for formation of a precipitate indicates the presence of phenolic content in the endophytes extract.

Ferric chloride test- Few drops of 5% FeCl3 was added to 2-3ml of extract and observed for deep blue black color indicates the presence of phenolic content.

6. Determination of Glycosides

The different extracts were hydrolysed with 5 ml conc. HCl for 2 hours on a water bath and filter. The hydrolysate was subjected to the following tests. **Borntrage's test** - In a test tube 2 ml of filtered hydrolysate were taken and into it added 3 ml of chloroform and mixed well. A chloroform layer was separated and to it 10% ammonia solution was added. A pink colour indicated the presence of glycosides

7. Determination of Terpenoids

Salkowski's test

This test is predominantly used for the determination of the terpenoids from the given samples. Chloroform (1 ml) was added to 1 ml of extract and a few drops of concentrated sulphuric acid were added along the sides of test tubes and observed for the formation of the yellow-colored layer indicating the presence of terpenoids.

8. Determination of Steroids

Tests for steroids

1 ml of bacteria and fungi ethyl solvent extracts was mixed with 1 ml of chloroform and add the 1 ml of sulphuric acid to the mixture on the sides of test tubes and observed for the formation of red colored upper layer complex. The red-colored upper layered complex indicates the presence of steroids.

4.3 RESULTS

Antioxidant activity- The DPPH and FRAP assays are two of the most common ones used to measure antioxidant capabilities. oxidative stress is widespread in chronic degenerative disease, dietary antioxidants may explain this protective effect. Every dietary plant contains a variety of antioxidants with varying characteristics.(Sethi *et al.*, 2020) Many of these antioxidants work together to reduce oxidative stress. Plants and endophytes together contribute to the production of antioxidant compounds (Blomhoff *et al*., 2006). Based on the results, DPPH free radical scavenging activity of endophytic fungal extracts ethyl acetate extracts shows a high free radical scavenging activity.(Iwansyah *et al*., 2019)

Antioxidant activity

According to the result of antioxidant activity, ethyl acetate extract shows a good result compared to other solvents the extract of B-45 endophyte bacteria shows high activity in all three assays The extract of endophyte fungi, F-14 shows high activity in the ethyl acetate solvent extract

Phytochemicals assay

The results of the phytochemicals assay showed the presence of alkaloids, flavonoids, saponins, tannins, phenol, and steroids in ethyl acetate extract of bacteria Culture B3 followed by B 45 overall, the ethyl acetate solvent is best for extraction of phytochemicals from the crude sample in case of fungi, F 14 and F 33 showed, the presence of phenol, flavonoids, tannins glycosides, which confirms there is an active component in it which may act as anti-microbial compounds. All the results are tabulated and analysed

4.4 DISCUSSION

The chapter five deals with the free radical scavenging activity and the phytochemical constituents of the solvent extracts of endophytic bacteria and fungi.

It is generally acknowledged that oxidation plays a significant role in both the body and food. The metabolism of oxidative substances is necessary for cell survival. The generation of free radicals and other reactive oxygen species, which lead to oxidative alterations, is a secondary consequence of this dependency. More and more evidence points to these species' participation in a range of typical in vivo regulatory mechanisms.

CHAPTER V

Characterization of Bioactive compounds from Endophytic Bacteria and Fungi Isolated from Edible Bamboo Shoots.

5.1 INTRODUCTION

It has been shown that a vast array of endophytic fungus may create a multitude of 152 bioactive compounds with capabilities including those of antioxidant, anticancer, anti-diabetic, anti-inflammatory, and immunosuppressive (Prakash *et al.,* 2020)Muscodor vitigenus, a unique endophytic fungus, was identified by Daisy et al. in the year 2002. This fungus, when cultivated under the suitable cultural conditions, generates naphthalene, which is an insect repellant. Endophytic fungus generates a wide variety of metabolites with a wide range of structures. Several groups have been established for the categorization of these natural products; alkaloids (such as amines and amides, indole derivatives, pyrrolizidines, and quinazolines), steroids, terpenoids (sesquiterpenes and diterpenes), isocoumarins, quinones, flavonoids, phenyl (Tan and Zou, 2001 and Yu *et al.,* 2010).(Kumar *et al.*, 2017) (Rana *et al*., 2022) The chromatographic technique or preparative high-performance liquid chromatography may be used to separate these combinations of natural products into their component compounds. After the peaks have passed through the detector, they are available for

collection one at a time. GC-MS is an ideal technique for distinguishing the volatile constituents of extracts long chain, branched chain, hydrocarbons, alcohols, acids, esters, and so on (Sermakkani and Thangapandian, 2012). (Karthik and Jayashankar, 2023) The segmented components are recognised using the best detection method (Mass-Spectroscopy), which is performed by GC-MS.

The need for novel antimicrobials derived from natural sources is growing due to the rise in the incidence of human diseases that are resistant to antibiotics. Because they are thought to have a defence mechanism against pathogenic invasion, endophytic bacteria and fungi have become a viable source of novel antimicrobial compounds

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive compounds with potential for exploitation in a wide variety of medical, agricultural and industrial areas. Numerous antimicrobial metabolites from endophytic bacteria and fungi have been found including alkaloids, flavonoids, phenolic acids, steroids, terpenoids and peptides.

In this current objective, the extraction of antimicrobial compounds from the selected endophytic bacteria and fungi was a prominent aim of the study.

5.2 MATERIALS AND METHOD

Selection of effective endophytic bacteria and fungi strains

1. Antimicrobial activity
2. Antioxidant activity
3. Qualitative analysis of phytochemicals from different solvent extracts

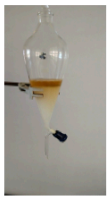
Endophytic bacteria strain
B45- *Bacillus velezensis*
NB

Endophytic fungi strain
F33- *Rigidoporus vinctus*
SDB

Mass culture production of secondary metabolite

Cell-free supernatant (CFS)

To CFS add an equal volume of ethyl acetate solvent in a separating funnel and agitated for 15min.



The organic phase was collected and the solvent was removed by evaporation under reduced pressure at 45 ° C using a rotary vacuum evaporator. The dried crude extracts were weighed, labelled in separate vials, and then stored at 4° C for further analysis

Partial purification of compounds by Preparative HPLC

Fractions are collected and subjected for biological assay

Antimicrobial assay

Minimum inhibitory concentration

Antioxidant assay

DPPH
FRAP
H_2O_2

Phytochemical assay

Total phenolic content

Total flavonoid content

LCMS analysis

Detection of the bioactive compound by GC-MS analysis

SEM Analysis to evaluate cell lysis and agglutination of bacteria cells

SEM Analysis To confirm the effect of antimicrobial compounds isolated from endophytes on the pathogen bacteria cell through visualizing by SEM

5.3 RESULTS AND DISCUSSION

Fraction eight of bacteria extract showed high positive result in antimicrobial assay, antioxidant assay Ethyl acetate solvent was used for extraction of bioactive compounds as it served as better solvent then methanol and chloroform. The extract was subjected for the partial purification by preparatory HPLC. Total 15 bacteria extract fractions and 7 fungi extract fractions are collected and they are tested for antimicrobial, antioxidant TPC, TFC By the above tests B Fr.8 and F Fr. 5 fraction shown significant antimicrobial property. Later the fractions are analysed for the compounds present in it by GC-MS and LC-MS B Fr.8 Fraction showed good biological activity compared to other. B Fr.8 fraction was found to disrupt the cell membrane of the target pathogen as evidenced by significant leakage of intracellular proteins and nucleic acids. B Fr.8 confirms it as a antimicrobial component which can be utilized as a active pharmaceutical compounds (API).

SUMMARY

This study aided to identify the endophytes which are highly potential in antimicrobial and antioxidant capacity which have capable of producing secondary metabolites consists of bioactive compound which gave a high positive approach for identification of the compound effort was made to identify and isolate the mixture of compound which are having high antimicrobial efficacy.

The outcome of this research

Total 137 bacteria isolate and 71 fungi cultures are obtained from eight different species of bamboo shoots from the Karnataka. Among 137 bacteria is isolates 19 isolate so good anti-microbial activity and out of 71 fungi cultures 15 cultures show excellent anti-microbial activity.

B3, B45, B65, B68 and B115 Endophytic bacteria CFS shows a remarkable activity which shows they have some antibacterial compound in it and B3, B45 and B68 shows moderate antifungal activity

F2, F14, F33, F45 and F62 Endophytic fungi CFS shows a remarkable activity which shows they have some antibacterial compound in it and F14 and F33 shows moderate antifungal activity The selected isolates were subjected for submerged fermentation for the production of bioactive compounds. The ethyl acetate solvent extract showed a good efficient activity in antimicrobial, antioxidant, and phytochemicals assays.

CONCLUSION

Total 8 species of bamboos were identified from 10 districts Karnataka region Effort was made to isolate endophytic bacteria and fungi from the bamboo shoots. By this study its confirm that endophytes are present in the bamboo shoots. A total 137 endophytic bacteria and 71 endophytic fungi were isolated.

Total 10 endophytes (5 endophytic bacteria and 5 endophytic fungi) were selected based on antimicrobial activity. The selected endophytes are molecularly identified and sequences are submitted to NCBI accession number is procured. Selected endophytes are subjected for qualitative analysis of phytocomponents and antioxidant activity. The positive strains F14 - *Rigidoporus vinctus* culture and B45- *Bacillus velezensis* strain are selected for the mass culture and production of secondary metabolites. Ethyl acetate solvent was used for extraction of bioactive compounds as it served as better solvent then methanol and chloroform. The extract was subjected for the partial purification by preparatory HPLC. Total 15 bacteria extract fractions and 7 fungi extract fractions are collected and they are tested for antimicrobial, antioxidant TPC, TFC. By the above tests B Fr.8 and F Fr. 5 fraction shown significant antimicrobial property. Later the fractions are analyzed for the compounds present in it by GC-MS and LC-MS. B Fr.8

Fraction showed good biological activity compared to other. B Fr.8 fraction was found to disrupt the cell membrane of the target pathogen as evidenced by significant leakage of intracellular proteins and nucleic acids. B Fr.8 confirms it as a antimicrobial component which can be utilized as an active pharmaceutical compounds (API).

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RESEARCH PUBLICATIONS

1. **Karthik. M.B** and Jayashankar. M, (**2023**) "**Nutritional and Qualitative Phytochemical Analysis of Edible Bamboo Shoots Harvested from Karnataka**" *International Journal of Food and Nutritional Sciences*, vol.12, S. Iss.1, PP. 1-11

2. **Karthik. M.B** and Jayashankar. M, (**2023**) ***"In Vitro* Antibacterial Efficacy and Molecular Identification of Endophytic Bacteria Isolated From *Dendrocalamus asper* and *Dendrocalamus strictus"*** *Eur. Chem. Bull.* ***2023****, 12(1),PP. 01 –11*

3. **Karthik. M.B** and Jayashankar. M, (**2023**) **"Rigidoporus vinctus, an endophytic fungus with antioxidant and antimicrobial properties, from the shoot of Bambusa tulda"** *Recent Research in Agriculture and Plant Sciences.* ISBN: 978-93-91248-70-3, PP. 50-60

4. Jayashankar. M, **Karthik. M.B** and Shradha. P.S (**2023**) **"Antibacterial potential of MBKF16 Endophytic fungi strain on clinical pathogens"** *European Journal of Molecular & Clinical Medicine*. Vol.10, Iss.(8), PP. 291-299

WORKSHOPS ATTENDED

1. Participated in the **'Seven Days National Level Training Programme' of the DST-STUTI-2022** held during November 14th to 20th, 2022 the programme was organized by the DST-PURSE Instrumentation Centre, CARRT and MHRD-RUSA MMR Instrument Centre

Mangalore University, Mangalagangothri, Karnataka. in collaboration with Shivaji University Kolhapur Maharashtra.

2. Participated in the **'Seven Days Online International Workshop on advance statistical data analysis using SPSS'** held on January 21st to 27th, 2022 the programme was organized by Science Tech Institute, Lucknow, UP India.

3. Participated in **Ten Days workshop on** "**Application of Artificial Intelligence in Bioinformatics and Biomedical Science"** held on 27th January to 6th February 2021. the programme was organized by Karnataka State Akkamahadevi Women's University Vijayapura Department of Bioinformatics under CURIE-AI, DST, New Delhi

4. Participated in the "**Three Days Online Workshop on Advancement in Applied Microbiology and Biotechnology 2021"** held on 15th to 17th February 2021. Organized by the Department of Microbiology Mangalore University Jnana Kaveri Campus Chikka Aluvara Kodagu Karnataka India in association with the BIOSOL, Punjab, India

PAPERS PRESENTED IN CONFERENCES

1. **Karthik. M.B** and M. Jayashankar ***"Molecular Identification of Endophytic Bacteria from Bamboo - A Poaceae Family"*** Presented in International conference on Recent Advancement in Chemistry organized by Department of Chemistry Field Marshal K M Cariappa College Mangalore University on Wednesday, 23rd November **2022**

2. **Karthik. M.B** and M. Jayashankar "***Molecular Identification of endophytic fungi and its antimicrobial, antioxidant ability from edible shoots of Bambusa species***" Presented in 62nd Annual International conference of Association of Microbiologist of India (AMI) conducted by University of Mysore on 21st to 23rd. September **2022**

3. **Karthik. M.B** and M. Jayashankar "***Isolation and Molecular identification of endophytic bacteria from edible bamboo shoots of Kodagu region***" presented in National conference on Advances in Microbial Biotechnology: Current Trends and Future prospects (AMBT-2022) organized by Department of Microbiology, Biotechnology and Food technology, Bangalore University, 28th and 29th April, **2022**

4. **Karthik. M.B** and M. Jayashankar "***Molecular identification of Endophytes from edible shoots of Dendrocalamus asper and Dendrocalamus stocksii.***" presented in the National conference Integrated Microbial Technology for Sustainable Future (IMTSF-2022) organized by the Department of Studies in Microbiology, Mangalore University. and Microbiologists' Society India. PEARL-A Foundation for Educational Excellence on 7th and 8th July, **2022**

5. **Karthik. M.B** and M. Jayashankar ***"Cerrena unicolor* - A Endophytic fungi isolated from shoot of *Bambusa tulda* its phytochemicals, antioxidant and antagonistic activity on clinical pathogens"** presented in the National conference on Advances in Biotechnology and Nanotechnology organized by the P G Department of Studies in Microbiology and Biotechnology Karnataka University, Dharwad. held on 14th and 15th March **2023**

6. **Karthik. M.B** and M. Jayashankar "***Rigidoporus vinctus*, an endophytic fungus with antioxidant and antimicrobial properties, from the shoot of *Bambusa tulda*"** presented in the 2nd International Conference on Advance Interdisciplinary Research (ICAIR-2023) Jointly Organised by Digvijay Nath Post Graduate College, Gorakhpur, UP and Science Tech Institute, Lucknow held on 7th to 9th April **2023**

Signature of Research guide **Signature of Candidate**